View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Keele Research Repository

Cumulin and FSH cooperate to regulate inhibin B and activin B production by human granulosa-lutein cells in vitro

Dulama Richani, Katherine Constance, Shelly Lien, David Agapiou, William A. Stocker, Mark P. Hedger, William L. Ledger, Jeremy G. Thompson, David M. Robertson, David G. Mottershead, Kelly L. Walton, Craig A. Harrison and Robert B. Gilchrist

Endocrinology
Endocrine Society

Submitted: December 05, 2018
Accepted: February 01, 2019
First Online: February 08, 2019

Advance Articles are PDF versions of manuscripts that have been peer reviewed and accepted but not yet copyedited. The manuscripts are published online as soon as possible after acceptance and before the copyedited, typeset articles are published. They are posted "as is" (i.e., as submitted by the authors at the modification stage), and do not reflect editorial changes. No corrections/changes to the PDF manuscripts are accepted. Accordingly, there likely will be differences between the Advance Article manuscripts and the final, typeset articles. The manuscripts remain listed on the Advance Article page until the final, typeset articles are posted. At that point, the manuscripts are removed from the Advance Article page.

DISCLAIMER: These manuscripts are provided "as is" without warranty of any kind, either express or particular purpose, or non-infringement. Changes will be made to these manuscripts before publication. Review and/or use or reliance on these materials is at the discretion and risk of the reader/user. In no event shall the Endocrine Society be liable for damages of any kind arising references to, products or publications do not imply endorsement of that product or publication.

ADVANCE ARTICLE: Endocrinology

ENDOCRINE SOCIETY

Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

Endocrinology; Copyright 2019 DOI: 10.1210/en.2018-01026

Cumulin and FSH jointly regulate inhibin/activin B

Cumulin and FSH cooperate to regulate inhibin B and activin B production by human granulosa-lutein cells in vitro

Dulama Richani*[1], Katherine Constance[1], Shelly Lien[1], David Agapiou[1], William A. Stocker[2,3], Mark P. Hedger[4], William L. Ledger[1], Jeremy G. Thompson[5], David M. Robertson[1], David G. Mottershead[5,6], Kelly L. Walton[2], Craig A. Harrison[2] and Robert B. Gilchrist[1]

[1] *Fertility and Research Centre, School of Women's and Children's Health, University of New South Wales Sydney, Australia*

[2] *Department of Physiology, Monash Biomedicine Discovery Institute, Monash University, Clayton, Australia*

[3] *Department of Chemistry and Biotechnology, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia*

[4] *Centre for Reproductive Health, Hudson Institute of Medical Research, Clayton, VIC, Australia*

[5] *Robinson Research Institute, Adelaide Medical School, The University of Adelaide, Australia*

[6] *Institute for Science and Technology in Medicine, School of Pharmacy, Keele University, Newcastle-under-Lyme, UK*

ORCiD numbers:

0000-0003-1886-0423

Richani

Dulama

0000-0003-1611-7142

Gilchrist

Robert B.

Received 05 December 2018. Accepted 01 February 2019.

The oocyte-secreted factors bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) interact functionally and it is hypothesised that this interaction may be mediated by formation of a GDF9:BMP15 heterodimer, termed cumulin. GDF9 and BMP15 regulate folliculogenesis and ovulation rate, and have been shown to regulate inhibin and activin, local regulators of folliculogenesis. The objective of this study was to determine if cumulin regulates granulosa cell inhibin and activin production, and if this requires cooperation with FSH. Human granulosa-lutein (hGL) cells collected from IVF patients were cultured ± FSH with various forms of recombinant cumulin (native and cysteine mutants, and with/without the pro-domains), and cysteine mutant GDF9 or BMP15. Messenger RNA expression of the subunits of inhibins/activins (*INHA*, *INHBA*, *INHBB*) and secretion of inhibin A, inhibin B, and activin B were measured. Mature- and pro-forms of cumulin stimulated comparable *INHBB* mRNA expression and secretion of inhibin B and activin B, whereas GDF9 or BMP15 exhibited no effect. Cumulin, but not GDF9 or BMP15, interacted synergistically with FSH to increase *INHBB* mRNA and inhibin B expression. FSH markedly stimulated *INHA*, which encodes the α subunit of inhibin A/B, and suppressed activin B. Cumulin ± FSH did not significantly alter inhibin A. Together these data demonstrate that cumulin, but not GDF9 or BMP15, exerts paracrine control of FSH-induced

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
ADVANCE ARTICLE
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

regulation of inhibin B and activin B. The pro-domains of cumulin may have a minimal role in its actions on granulosa cells.

INTRODUCTION

The pituitary gonadotropins, FSH and LH stimulate folliculogenesis by promoting growth and differentiation of the granulosa cells of the ovarian follicle[1]. In addition, local growth factors produced by ovarian follicular cells and the oocyte itself regulate follicle development through their direct actions on granulosa cells and also indirectly by modulating gonadotropin secretion. Belonging to the TGFβ superfamily, activins and inhibins exert opposing effects on pituitary FSH production and secretion, whereby they stimulate and perturb FSH production, respectively [2]. FSH and LH actions in the follicle are also regulated by the oocyte-secreted growth factors; growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), and this interaction between gonadotropins and oocyte paracrine factors is thought to underpin the basic physiological mechanism regulating the species-specific ovulation rate and fecundity in mammals [3-5].

Over the past decade it has become clear that the oocyte plays a major role in regulating follicle development and modulating its own follicular microenvironment by the secretion of paracrine growth factors that act on the follicle somatic cells [6]. GDF9 and BMP15 are two closely related paralogs of the TGFβ superfamily that are principally produced by the oocyte in females [7]. These two oocyte-secreted factors act on surrounding granulosa cells to regulate their growth and differentiation, affecting folliculogenesis and oocyte development and maturation. GDF9 and BMP15 are essential for female fertility, and genetic mutations in these growth factors alter follicle development and thus reproductive potential in mammals (reviewed by Juengel, McNatty [8]). GDF9 and BMP15 signal via BMPRII/ALK cell surface receptors to phosphorylate intracellular SMAD proteins that regulate the transcription of several genes [4].

Like all TGFβ superfamily ligands, GDF9 and BMP15 are produced as pro-mature proteins and require proteolytic cleavage by furin-like proteases to release the N-terminal pro-domain from the receptor binding C-terminal mature domain to become bioactive [9,10]. However, GDF9 and BMP15 are unusual TGFβ ligands as they lack the conserved fourth cysteine residue required to make the intersubunit disulphide bond that links TGFβ ligand dimers covalently, and therefore they form non-covalent dimers [11]. Due to their non-covalent dimer interaction, shared spatiotemporal expression pattern in the oocyte, close structural homology, and their co-immunoprecipitation, it is speculated that GDF9 and BMP15 can physically interact, likely to form a GDF9:BMP15 heterodimer, called cumulin [4,12-14]. Functional studies have shown potent GDF9+BMP15 synergistic responses in granulosa cell proliferation, progesterone and inhibin α subunit protein production, and SMAD activity in vitro [14-16], and such GDF9+BMP15 synergism is thought to be due to the formation of a structural heterodimer of GDF9 and BMP15, i.e. cumulin [12]. Recently, we synthesised cumulin, modelled its receptor interactions and signaling, demonstrated its high potency on granulosa cells, and hypothesized a central role for cumulin in determining the low ovulation rate phenotype in mammals such as humans [12].

Due to its recent characterization, most aspects of cumulin's molecular form and cellular and physiological functions require elucidation. Nonetheless, new evidence suggests cumulin has significant potential for improving the treatment of female infertility. Pro-cumulin is able to improve the quality and developmental competence of pig and human oocytes matured in vitro [12,17]. Interestingly, these improvements were only elicited by the pro-cumulin form and not by the highly bioactive mature form which has no pro-region [12]. This suggests that the GDF9 and BMP15 pro-domains in cumulin play an important role in its function [18,19]. Mouse pro-GDF9 induces inhibin B production by human granulosa-lutein cells ([20]) and pro-GDF9

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
ADVANCE ARTICLE
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

and pro-BMP15 potently synergize to promote total inhibin (including the bioinactive free α subunit monomer) production by sheep granulosa cells [15], leading us to hypothesize a role for pro-cumulin in the inhibin-activin system in granulosa cells.

The objectives of this study were to examine the role of cumulin, including the role of its pro-domains, and its cooperative effects with FSH, on granulosa cell production of inhibins and activins. To do this, various recombinant forms of cumulin dimers that were covalently or non-covalently linked, with and without their pro-domain, were generated and their effect on human granulosa-lutein cell production of inhibin A and B and activin B were examined.

MATERIALS AND METHODS

Human granulosa-lutein cell purification and culture

This study was approved by IVF Australia Ethics Committee (approval number 100). Human granulosa-lutein (hGL) cells were purified from follicular aspirates of women undergoing transvaginal oocyte retrieval following controlled ovarian stimulation for in vitro fertilisation. Follicle aspiration was performed 36 hours after administration of recombinant human chorionic gonadotropin (Ovidrel) to induce final oocyte maturation. For each experiment, cells from at least four patients were pooled and no patient samples were excluded from the study. Cells were prepared using a modified version of the methods described by Ferrero et al. (2012)[21] and Chang et al. (2013)[22]. Briefly, follicular fluid was passed through a 70µm cell strainer (Corning, Corning, USA) that was back washed with media to collect cell aggregates, which were recovered following centrifugation at 400xg for 10 min. The cell pellet was resuspended in HEPES-buffered αMEM (Gibco, Waltham, USA) containing 0.3% BSA (CellMaxx, MP Biomedicals, New Zealand) and hyaluronidase (100 mg/mL), followed by centrifugation at 1000xg for 2 min. The pellet was then resuspended in 4ml media and layered onto 8ml of Ficoll-Paque Premium 1.084 (GE Healthcare, Chicago, USA), centrifuged at 1000xg for 25 min and the interphase was collected. Cells were washed in DMEM (Gibco, Waltham, USA) containing 5% FBS (Gibco, Waltham USA) and 1% (v/v) Antibiotic Antimycotic Solution (Gibco, Waltham USA) and plated in the same media at a density of $3x10^5$ cells per well in 1mL in a 24 well plate (Costar, Corning, USA). Cells were incubated for 5 days at 37ºC in 5% CO_2 with media changes every second day to re-derive FSH-sensitive granulosa cells as previously described[23-25]. After 120 h, cells were washed once with 1 mL DMEM containing Antibiotic Antimycotic Solution and 0.3% BSA, and treated for 24 h with 500 µL of this media containing the various growth factors (see next section and Figure 1) ± 50 mIU/mL recombinant human FSH (Follitropin-β, Puregon, Organon, Oss, The Netherlands), as indicated in the text and figure legends. Cell pellets or conditioned media were then used for qPCR or ELISA assays, respectively.

Recombinant proteins

GDF9, BMP15 and cumulin in their pro-mature dimeric forms and mature cumulin (Figure 1 and Table 1) were used in this study. A pro-form refers to a dimeric complex of proteolytically cleaved pro- and mature-domain forms (Fig. 1A-D), whereas the mature form is a dimer lacking the pro-domain (Fig. 1E). As GDF9 and BMP15 exist as non-covalent dimers lacking the fourth cysteine residue common in TGFβ superfamily members, Ser^{418} of GDF9 and Ser^{356} of BMP15 were modified by substituting to cysteines, as previously described, to generate stabilised covalent-linked dimers of GDF9, BMP15 and cumulin that cannot dissociate [12,16]. These covalent proteins are indicated by the yellow link on the mature dimer in figures 1A-C, 1E. All proteins were produced in-house and human protein sequences were used.

Production and purification of non-covalent pro-cumulin and covalent mature cumulin (Fig. 1D-E) was carried out as previously described [12]. Their bioactivities were assessed based on their ability to activate SMAD signalling pathways as measured by SMAD-

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

responsive luciferase assays (A3-luciferase and BRE-luciferase) in the COV434 human granulosa cell line, as previously described [12].

Production and purification of pro-mature complexes containing covalently stabilised dimeric mature domains (pro-GDF9^{S418C}, pro-BMP15^{S356C} and covalent pro-cumulin; Fig. 1A-C) were produced similarly to that described previously [12]. Differences in methodology included production of recombinant proteins by transient transfection of HEK-293T cells using polyethylenimine-MAX (PEI-MAX) (Polysciences, Warrington, PA). When producing pro-GDF9^{S418C} or pro-BMP15^{S356C} homodimers, one plasmid type was transfected containing the respective cDNA. To produce the heterodimer covalent pro-cumulin, plasmids for both GDF9^{S418C} and BMP15^{S356C} were co-transfected. Protein purification was undertaken by cobalt-based immobilized metal affinity chromatography (Co-IMAC) using HisPur™ Cobalt Resin (Thermo Fisher Scientific, Waltham, MA). Bound proteins were eluted from Co-IMAC resin using elution buffer (50 mM phosphate buffer, 300 mM NaCl, 200 mM imidazole, pH 7.4). Imidazole was removed by dialysis against binding buffer (50 mM phosphate buffer, 300 mM NaCl, pH 7.4) using Slide-A-Lyzer® MINI Dialysis Devices (2 mL 3.5K MW Cut-off; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's guidelines. Activation of SMAD signalling reporters, A3-luciferase and BRE-luciferase, was assessed in the COV434 human granulosa cell line as previously described [12].

Real-time RT-PCR

Total RNA was isolated from cell pellets using the Qiagen microRNEasy kit (Qiagen, Germany) per the manufacturer's protocol. Total RNA was measured using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, USA). First-strand cDNA was synthesised using Superscript III reverse transcriptase and random primers (Invitrogen, Waltham, USA). Real-time RT-PCR was performed in duplicate using gene primers (GeneWorks, Australia; Table 2) on a Roche LightCycler 480 (Roche, Basel, Switzerland). Controls, including the absence of cDNA template or the reverse transcriptase enzyme in otherwise complete reactions showed no product amplification or genomic DNA contamination. Target gene expression was normalised to *GAPDH* expression. Using the statistical program BestKeeper, *GAPDH* was determined as a stable housekeeping gene with a SD (±Ct) and CV (%Ct) of 0.43 and 2.37, respectively, when its expression was measured across 5 different pools of hGL cells treated ± pro-GDF9, pro-BMP15, and pro-cumulin, all ± FSH. PCR data is expressed as the raw gene expression level using the $2^{-(\Delta CT)}$ method [26].

Inhibin A, B and Activin B ELISAs

Inhibin A and B are heterodimers of a common α-subunit bound covalently to a βA- or βB-subunit, respectively. The pro-forms of inhibin A and inhibin B were measured in hGL cell conditioned media using immunoassays as detailed by Walton et al. (2013)[27] that utilise antibodies to the α-, βA- and βB-subunits of inhibin.

Activin B is a homodimer of the βB-subunit. Total activin B was measured in hGL cell conditioned media using the Ansh Labs commercial ELISA kit (Ansh Labs, Webster, TX) according to the manufacturer's instructions, or using an in-house ELISA possessing similar sensitivity and specificity for human activin B, as previously described [28]. Samples that gave readings below the limit of quantification (LOQ) of the assay were assigned values of LOQ/2.

Statistical analyses

Results are presented as the mean ± SEM. The number of independent pools of granulosa cells tested for each experiment is indicated in the figure legends. Statistical analyses were performed using GraphPad Prism 7.02 software and differences were considered to be significant at $P \leq 0.05$. Data that were not normally distributed were transformed before

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
ADVANCE ARTICLE
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

analysis using log transformation. One-way ANOVA or two-way ANOVA (where two independent variables were tested) with multiple comparisons tests were used.

RESULTS

Cumulin stimulates granulosa cell inhibin B production

Covalent mature cumulin and non-covalent pro-cumulin each dose-dependently increased *INHBB* mRNA expression (Fig. 2A), which encodes a βB-subunit that in homodimeric form makes activin B or as a heterodimer with an α-subunit forms inhibin B. *INHBA* or *INHA* mRNA expression (encoding the βA- and α-subunits, respectively) was unaffected by treatment with either form of cumulin (Fig. 2B-C). Covalent pro-cumulin stimulated a significant dose-dependent increase in *INHBB* expression relative to no treatment control, however treatment with either of the homodimers, covalent pro-GDF9 and covalent pro-BMP15, did not affect *INHBB* mRNA expression at any dose tested (Fig. 3A). None of the proteins elicited a significant increase in *INHBA* (Fig. 3B) or *INHA* (Fig. 3C) mRNA expression, except *INHBA* which was significantly higher than control following exposure to 12.5 ng/mL covalent pro-cumulin (Fig. 3B).

Cumulin and FSH exert dual control of inhibin B production

FSH, a known driver of inhibin secretion in granulosa cells [29] gave a marked 15-fold increase in *INHA* mRNA expression (Fig. 4A). The FSH effect on *INHA* mRNA expression was further significantly ($P<0.05$) enhanced by co-treatment with pro-BMP15, or pro-cumulin but not pro-GDF9. Pro-cumulin and FSH synergistically increased (i.e. the effect of both factors combined was greater than the sum of either factor alone) *INHBB* mRNA and inhibin B protein production (two-way ANOVA, $P <0.05$; interaction of main effects of FSH and growth factor), whilst pro-GDF9 ± FSH and pro-BMP15 ± FSH had no significant effect (Figs. 4B, C). A trend in interactive effect between FSH and treatment on activin B was observed ($P =0.07$; Fig. 4D). Activin B production was upregulated by pro-cumulin, but only in the absence of FSH, whilst pro-GDF9 ± FSH and pro-BMP15 ± FSH had no significant effect on activin B (Fig. 4D).

The pro-domain does not affect hGL cell responsiveness to cumulin

As the pro-domains of GDF9, BMP15 and cumulin are required for the stimulatory effect of these growth factors on oocyte quality, as measured by an oocyte's capacity to support development to the blastocyst stage [12,18,19], a comparison was made between pro-cumulin and mature cumulin. Whilst pro- and mature-cumulin both significantly (two-way ANOVA, $P \leq 0.01$; main effects) promoted *INHBB*, inhibin B and activin B levels, no significant difference was observed between them in terms of the extent of stimulation at the tested dose (Fig. 5). Overall, FSH stimulated inhibin B production while suppressing activin B (2-way ANOVAs, $P <0.01$; main effects of FSH) (Fig. 5B, 5C).

Inhibin A production is not regulated by cumulin

Pro-cumulin in the presence of FSH stimulated *INHBA* mRNA expression whilst mature cumulin did not (Fig. 5D). Inhibin A secretion in granulosa cell cultures was unaffected by FSH, by either form of cumulin or by the interaction of FSH and cumulin (Fig. 5E).

DISCUSSION

The nature of the native forms of GDF9 and BMP15, and whether they occur physiologically as the heterodimer cumulin, remains unclear as they are yet to be isolated from biological fluids. However, there is substantial evidence of synergistic interactions between GDF9 and BMP15, and a prominent hypothesis in the field is that such synergism is mediated by a heterodimer molecule, cumulin, consisting of at least one subunit of GDF9 and BMP15 [12]. In

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019
ADVANCE ARTICLE

this study, we demonstrate that cumulin regulates inhibin B and activin B production in human granulosa-lutein cells. We bioengineered and purified wild-type non-covalent cumulin, as well as covalent dimers containing an introduced inter-subunit disulphide bridge. Pro- and mature-cumulin induced comparable expression of *INHBB*, inhibin B, and activin B in hGL cells, whereas covalent pro-GDF9 or pro-BMP15 elicited no effect. This supports that GDF9 and BMP15 linkage results in distinct activation of gene expression, relative to GDF9 or BMP15 alone.

These observations are in line with a previous study showing synergistic upregulation of total inhibin (including the bioinactive free α subunit monomer) in rat granulosa cells when murine or ovine GDF9 conditioned media was added together with ovine BMP15 conditioned media, but no effect was seen when either GDF9 or BMP15 conditioned media were used in isolation [15]. Moreover, others have reported a negative effect of GDF9 or BMP15 alone on inhibin production. Human BMP15 had no effect on *INHA*, *INHBA*, and *INHBB* mRNA expression in rat granulosa cells, and it decreased mRNA expression of these subunits in the presence of FSH [30]. Mouse GDF9 also inhibited total inhibin production in ovine and bovine granulosa cells [31], and E.coli expressed human GDF9 had no effect on inhibin A and B production in hGL cells [32]. In contrast to these studies, GDF9 and BMP15 individually have been reported to regulate the inhibin system in granulosa cells. Pulkki *et al.* (2011, 2012) [33,34] demonstrated that purified mature human BMP15 was a stimulator of inhibin B production in hGL cells. Roh *et al.* (2003) reported upregulation of inhibin A and inhibin B in rat granulosa cells in response to mature rat GDF9, an effect that was enhanced by FSH [35]. Kaivo-Oja *et al.* (2003) also reported stimulation of inhibin B secretion by mature rat GDF9 in hGL cells [20]. These discrepancies in the literature on the roles of GDF9 and BMP15 in regulating inhibin production are likely due to methodological differences such as the use of different granulosa cell types from different species at different stages of folliculogenesis, and the large variability in the construct and purity of the in-house produced growth factors used. Many of the early studies in the field used unpurified growth factors including raw conditioned media and this is likely to be a significant contributor to the discrepancy in reporting. Indeed, this issue persists in the case of cumulin as it is newly described and there are just three publications to date [12,13,36]. In this study we generated highly purified forms of cumulin, including novel variants such as covalent pro-cumulin, which has enabled further scrutiny of its role in regulating granulosa cell function. Overall, the findings of this study demonstrate that cumulin regulates granulosa cell inhibin B and activin B production, and the homodimers GDF9 or BMP15 alone do not. There was no pronounced effect of cumulin on inhibin A production despite the stimulatory effect on inhibin B. This is in line with previous work where two other Smad2/3-activating members of the TGFβ superfamily, TGFβ1 and activin A, were shown to have a more marked stimulatory effect on inhibin B than on inhibin A production in rat granulosa cells [37].

FSH is a well-established stimulator of granulosa cell inhibin subunit gene expression and inhibin A and B production [37,38]. FSH markedly upregulated *INHA* expression that encodes the α subunit of inhibin A/B, and expression was further enhanced by BMP15 and cumulin. Moreover, a synergistic interaction was seen between FSH and covalent pro-cumulin in upregulating inhibin B. FSH also decreased activin B production. Hence, we postulate that cumulin and FSH synergistically increase inhibin B production by increasing the availability of the βB and α subunits, respectively, leading to heterodimerisation of the α and βB subunits to form inhibin B. This occurs at the expense of activin B production since activin B is comprised of two βB subunits. No synergistic interaction was observed between FSH and either GDF9 or BMP15 alone, providing evidence to support the hypothesis that the basis for their regulation of the inhibin-activin system is through the formation of cumulin; the use of covalently linked mutant dimers of cumulin provides further support that they can act as a

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

functional dimeric unit. Whether cumulin exists naturally as a free dimeric entity, or GDF9 and BMP15 form a heterodimer at the receptor level remains unclear. Using Western blotting procedures, Heath *et al.* (2017) recently reported that the predominate forms of GDF9 and BMP15 secreted by ovine and bovine oocytes were cleaved and uncleaved pro-mature monomers, and hypothesised that these monomers form a GDF9:BMP15 heterodimer at the receptor level [36].

Previous investigation into the effects of cumulin on oocyte quality showed that cumulin's pro-domain was important for cumulin to elicit an increase in subsequent embryo yield of in vitro matured porcine cumulus-oocyte complexes [12]. This supported previous findings that pro-BMP15, but not mature BMP15 or mature GDF9, improve oocyte quality [18,19], suggesting that the pro-domain plays an important role in its function on cumulus cells. Hence we investigated the effect of the cumulin pro-domain on the inhibin-activin system using cumulin forms with and without the pro-domain. Mature covalent cumulin and non-covalent pro-cumulin elicited comparable expression levels of *INHBB*, inhibin B and activin B, suggesting that the disulphide link and pro-domain do not affect granulosa cell responsiveness to cumulin. It is intriguing that the pro-domain plays a crucial functional role on the cumulus-oocyte complex but not on granulosa cells. The current hypothesis underpinning this is that the pro-domains of GDF9/BMP15/cumulin interact with the specialised heparin-sulphated proteoglycans of the cumulus-oocyte complex facilitating in some manner distinct ligand-receptor signalling cascades between the two cell types [39].

There is compelling genetic evidence that paracrine signals from the oocyte in the form of GDF9 and BMP15 (and possibly cumulin), constitute key regulators of folliculogenesis, ovulation rate and fecundity in mammals [4]. This notion is bolstered by the results of the current study whereby cumulin and FSH interact to enhance inhibin B production at the expense of activin B, as inhibin is a key endocrine regulator of FSH. In humans, inhibin B is the predominate form of inhibin/activin produced by granulosa cells in the early follicular phase [40]. The current results suggest that oocyte paracrine signals, likely in the form of GDF9/BMP15/cumulin, cooperate with FSH to induce inhibin B during the follicular phase, which in turn feeds back to suppress pituitary FSH secretion, which limits the growth of multiple Graafian follicles and hence the ovulation quota. Oocyte paracrine signals including cumulin may have different effects on granulosa cells from other stages of folliculogenesis, e.g. inhibin A or activin production may be favoured. Oocyte cumulin-mediated enhancement of inhibin and corresponding antagonism of circulating FSH may be a feature of low-ovulation phenotype mammals such as humans, as oocytes of polyovular mammals such as mice produce little BMP15 [41] and hence presumably little cumulin [12]. Consequently, it can be speculated that the cooperative regulation of inhibin B by cumulin and FSH may play a role in regulating folliculogenesis and fecundity in women.

ACKNOWLEDGEMENTS

We are grateful to the team of embryologists at IVF Australia for collecting the hGL cells used in this study. We thank Karen Chan and Susan Hayward for their assistance with ELISAs. We thank Mark Donoghoe from Stats Central, Mark Wainwright Analytical Centre, UNSW Sydney, for his general statistical advice. The Hudson Institute is supported in part by the Operational Infrastructure Support Program of the Victorian State Government, Australia.

Funding: The work was funded by National Health and Medical Research Council of Australia grants (APP1017484, APP1024358, APP1121504 awarded to DGM, CAH, and RBG, respectively) and fellowships (APP1023210, APP1117538 awarded to RBG), and by Strategic Funds from the University of New South Wales Sydney awarded to RBG. WAS was supported by a Research Training Program stipend from the Department of Education and Training, Australia.

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019
ADVANCE ARTICLE

National Health and Medical Research Council http://dx.doi.org/10.13039/501100000925, APP1017484, David G. Mottershead; National Health and Medical Research Council http://dx.doi.org/10.13039/501100000925, APP1024358, Craig A. Harrison; National Health and Medical Research Council http://dx.doi.org/10.13039/501100000925, APP1121504, Robert B. Gilchrist; National Health and Medical Research Council http://dx.doi.org/10.13039/501100000925, APP1023210, Robert B. Gilchrist; National Health and Medical Research Council http://dx.doi.org/10.13039/501100000925, APP1117538, Robert B. Gilchrist; University of New South Wales Sydney, Robert B. Gilchrist; Department of Education and Training http://dx.doi.org/10.13039/501100007912, William A. Stocker

Corresponding author: *Dulama Richani (d.richani@unsw.edu.au), Wallace Wurth Building, UNSW Sydney, Kensington, NSW 2052, Australia

Re-print requests: Dulama Richani (d.richani@unsw.edu.au), Wallace Wurth Building, UNSW Sydney, Kensington, NSW 2052, Australia

Disclosure:
The authors have nothing to disclose.

REFERENCES

1. Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet.* 1997;15(2):201-204.
2. Walton KL, Makanji Y, Robertson DM, Harrison CA. The synthesis and secretion of inhibins. *Vitam Horm.* 2011;85:149-184.
3. McNatty KP, Juengel JL, Pitman JL. Oocyte-Somatic Cell Interactions and Ovulation Rate: Effects on Oocyte Quality and Embryo Yield. *Reprod Biol Insights.* 2014;7:1-8.
4. McNatty KP, Moore LG, Hudson NL, et al. The oocyte and its role in regulating ovulation rate: a new paradigm in reproductive biology. *Reproduction.* 2004;128(4):379-386.
5. Findlay JK, Dunning KR, Gilchrist RB, Hutt KJ, Russell DL, Walters KA. Follicle selection in mammalian ovaries. In: Leung PCK, Adashi EY, eds. *The Ovary*. 3 ed. London: Academic Press 2018:3-21.
6. Gilchrist RB. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. *Reprod Fertil Dev.* 2011;23(1):23-31.
7. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update.* 2008;14(2):159-177.
8. Juengel JL, McNatty KP. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Hum Reprod Update.* 2005;11(2):143-160.
9. Al-Musawi SL, Walton KL, Heath D, Simpson CM, Harrison CA. Species differences in the expression and activity of bone morphogenetic protein 15. *Endocrinology.* 2013;154(2):888-899.
10. Simpson CM, Stanton PG, Walton KL, et al. Activation of latent human GDF9 by a single residue change (Gly 391 Arg) in the mature domain. *Endocrinology.* 2012;153(3):1301-1310.
11. McPherron AC, Lee SJ. GDF-3 and GDF-9: two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. *The Journal of biological chemistry.* 1993;268(5):3444-3449.

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
ADVANCE ARTICLE
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

12. Mottershead DG, Sugimura S, Al-Musawi SL, et al. Cumulin, an Oocyte-secreted Heterodimer of the Transforming Growth Factor-beta Family, Is a Potent Activator of Granulosa Cells and Improves Oocyte Quality. *The Journal of biological chemistry.* 2015;290(39):24007-24020.
13. Peng J, Li Q, Wigglesworth K, et al. Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(8):E776-785.
14. McIntosh CJ, Lun S, Lawrence S, Western AH, McNatty KP, Juengel JL. The proregion of mouse BMP15 regulates the cooperative interactions of BMP15 and GDF9. *Biology of reproduction.* 2008;79(5):889-896.
15. McNatty KP, Juengel JL, Reader KL, et al. Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function. *Reproduction.* 2005;129(4):473-480.
16. Mottershead DG, Ritter LJ, Gilchrist RB. Signalling pathways mediating specific synergistic interactions between GDF9 and BMP15. *Molecular human reproduction.* 2012;18(3):121-128.
17. Gilchrist RB, Lolicato F, Romero S, et al. Cumulin and cAMP-modulators combined improve human oocyte in vitro maturation and embryo yield. Annual Meeting for the Society for Reproductive Biology; 2016; Brisbane Convention & Exhibition Centre, Australia.
18. Sudiman J, Ritter LJ, Feil DK, et al. Effects of differing oocyte-secreted factors during mouse in vitro maturation on subsequent embryo and fetal development. *J Assist Reprod Genet.* 2014;31(3):295-306.
19. Sudiman J, Sutton-McDowall ML, Ritter LJ, et al. Bone morphogenetic protein 15 in the pro-mature complex form enhances bovine oocyte developmental competence. *PloS one.* 2014;9(7):e103563.
20. Kaivo-Oja N, Bondestam J, Kamarainen M, et al. Growth differentiation factor-9 induces Smad2 activation and inhibin B production in cultured human granulosa-luteal cells. *J Clin Endocrinol Metab.* 2003;88(2):755-762.
21. Ferrero H, Delgado-Rosas F, Garcia-Pascual CM, et al. Efficiency and purity provided by the existing methods for the isolation of luteinized granulosa cells: a comparative study. *Human reproduction.* 2012;27(6):1781-1789.
22. Chang HM, Klausen C, Leung PC. Antimullerian hormone inhibits follicle-stimulating hormone-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells. *Fertility and sterility.* 2013;100(2):585-592 e581.
23. Kaivo-Oja N, Mottershead DG, Mazerbourg S, et al. Adenoviral gene transfer allows Smad-responsive gene promoter analyses and delineation of type I receptor usage of transforming growth factor-beta family ligands in cultured human granulosa luteal cells. *J Clin Endocrinol Metab.* 2005;90(1):271-278.
24. Vireque AA, Campos JR, Dentillo DB, et al. Driving Human Granulosa-Luteal Cells Recovered From In Vitro Fertilization Cycles Toward the Follicular Phase Phenotype. *Reprod Sci.* 2015;22(8):1015-1027.
25. Jaatinen R, Bondestam J, Raivio T, et al. Activation of the bone morphogenetic protein signaling pathway induces inhibin beta(B)-subunit mRNA and secreted inhibin B levels in cultured human granulosa-luteal cells. *J Clin Endocrinol Metab.* 2002;87(3):1254-1261.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25(4):402-408.

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
ADVANCE ARTICLE
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

27. Walton KL, Chan KL, Pruysers E, et al. Use of detergent-based buffers allows detection of precursor inhibin forms in an immunoassay format. *Mol Cell Endocrinol.* 2013;381(1-2):106-114.
28. Ludlow H, Phillips DJ, Myers M, et al. A new 'total' activin B enzyme-linked immunosorbent assay (ELISA): development and validation for human samples. *Clin Endocrinol (Oxf).* 2009;71(6):867-873.
29. Ying SY, Czvik J, Becker A, Ling N, Ueno N, Guillemin R. Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. *Proceedings of the National Academy of Sciences of the United States of America.* 1987;84(13):4631-4635.
30. Otsuka F, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *The Journal of biological chemistry.* 2001;276(14):11387-11392.
31. McNatty KP, Juengel JL, Reader KL, et al. Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function in ruminants. *Reproduction.* 2005;129(4):481-487.
32. Shi FT, Cheung AP, Leung PC. Growth differentiation factor 9 enhances activin a-induced inhibin B production in human granulosa cells. *Endocrinology.* 2009;150(8):3540-3546.
33. Pulkki MM, Mottershead DG, Pasternack AH, et al. A covalently dimerized recombinant human bone morphogenetic protein-15 variant identifies bone morphogenetic protein receptor type 1B as a key cell surface receptor on ovarian granulosa cells. *Endocrinology.* 2012;153(3):1509-1518.
34. Pulkki MM, Myllymaa S, Pasternack A, et al. The bioactivity of human bone morphogenetic protein-15 is sensitive to C-terminal modification: characterization of the purified untagged processed mature region. *Mol Cell Endocrinol.* 2011;332(1-2):106-115.
35. Roh JS, Bondestam J, Mazerbourg S, et al. Growth differentiation factor-9 stimulates inhibin production and activates Smad2 in cultured rat granulosa cells. *Endocrinology.* 2003;144(1):172-178.
36. Heath DA, Pitman JL, McNatty KP. Molecular forms of ruminant BMP15 and GDF9 and putative interactions with receptors. *Reproduction.* 2017;154(4):521-534.
37. Lanuza GM, Groome NP, Baranao JL, Campo S. Dimeric inhibin A and B production are differentially regulated by hormones and local factors in rat granulosa cells. *Endocrinology.* 1999;140(6):2549-2554.
38. Turner IM, Saunders PT, Shimasaki S, Hillier SG. Regulation of inhibin subunit gene expression by FSH and estradiol in cultured rat granulosa cells. *Endocrinology.* 1989;125(5):2790-2792.
39. Watson LN, Mottershead DG, Dunning KR, Robker RL, Gilchrist RB, Russell DL. Heparan sulfate proteoglycans regulate responses to oocyte paracrine signals in ovarian follicle morphogenesis. *Endocrinology.* 2012;153(9):4544-4555.
40. Groome NP, Illingworth PJ, O'Brien M, et al. Measurement of dimeric inhibin B throughout the human menstrual cycle. *J Clin Endocrinol Metab.* 1996;81(4):1401-1405.
41. Crawford JL, McNatty KP. The ratio of growth differentiation factor 9: bone morphogenetic protein 15 mRNA expression is tightly co-regulated and differs between species over a wide range of ovulation rates. *Mol Cell Endocrinol.* 2012;348(1):339-343.

Figure 1: The forms of GDF9, BMP15 and their heterodimer cumulin examined. Proteins were used in their respective pro-forms (A-D), consisting of a complex of pro- and mature-domains post-proteolytic cleavage, and cumulin was also used as a dimer of the mature domains lacking pro-domains (E). Covalent dimers containing an introduced inter-

ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
ADVANCE ARTICLE
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

subunit disulphide bridge are shown with a yellow link between the mature dimers (A-C, E). Human pro-GDF9 is naturally latent (A).

Figure 2: Dose response of pro-cumulin and mature cumulin on inhibin A and B subunit production. Human granulosa-lutein cells were treated for 24 h with non-covalent pro-cumulin or covalent mature cumulin ($cumulin_{s:c}$) at varying concentrations. Expression of *INHBB*, *INHBA*, and *INHA* mRNA was measured. Data represent mean ± SEM of 5 independent granulosa cell pools. ** P ≤0.01, *** P ≤0.001 vs control (0 ng/mL) for both pro-cumulin and mature cumulin treatments at the indicated dose. $^{\#}$ P <0.05 vs control (0 ng/mL) for pro-cumulin.

Figure 3: Comparative effect of pro-cumulin, pro-GDF9 and pro-BMP15 on inhibin subunit mRNA expression. Human granulosa-lutein cells were treated for 24 h with covalent forms of pro- cumulin, pro-GDF9 or pro-BMP15 at varying concentrations. Expression of *INHBB*, *INHBA*, and *INHA* mRNA was measured. Data represent mean ± SEM of 7 independent granulosa cell pools. * P ≤0.05, ** P ≤0.01, *** P ≤0.001 vs control (0 ng/mL) for pro-cumulin.

Figure 4: The interactive effects of FSH and cumulin on inhibin B and activin B production. Human granulosa-lutein cells were treated for 24 h without (control) or with 50 ng/mL covalent pro-GDF9, pro-BMP15 or pro-cumulin, all ± 50 mIU/mL FSH. Cellular mRNA expression of *INHBB* and *INHA*, as well as secreted inhibin B and activin B into conditioned media were measured. Data represent mean ± SEM of 5 independent granulosa cell pools. G^{*}, significant main effect of growth factor by two-way ANOVA; F^{*}, significant main effect of FSH by two-way ANOVA; GxF^{*}; significant main effect interaction between growth factor and FSH by two-way ANOVA. ns, no significant difference; Bars within a graph not sharing a common letter are statistically different (P <0.05, multiple comparisons test).

Figure 5: Effect of the cumulin pro-domain on inhibin A, inhibin B and activin B production. Human granulosa-lutein cell *INHBB* and *INHBA* mRNA expression (n=4), inhibin B (n=3), inhibin A (n=3), and activin B (n=5) protein secretion were measured following 24 h treatment with non-covalent pro-cumulin (50 ng/mL) or covalent mature cumulin (50 ng/mL), all ± FSH (50 mIU/mL). Bars within a graph not sharing a common letter are statistically different ($-FSH^{a-b}$, $+FSH^{x-y}$; P <0.05).

Table1: Description of the purified recombinant growths factors used in this study.

Protein	Covalent	Pro- domain
Cumulin$^{S418C, S356C}$	✓	X
Pro-cumulin	X	✓
Pro-cumulin$^{S418C, S356C}$	✓	✓
Pro- GDF9^{S418C}	✓	✓
Pro-BMP15^{S356C}	✓	✓

Table 2: PCR primer sequences used for real-time RT-PCR

Gene	GenBank accession	Forward primer (5'- 3')	Reverse primer (5'- 3')
INHA	NM_002191.2	GTCTCCCAAGCCATCCTTTT	TGGCAGCTGACTTGTCCTC
INHBA	NM_002192.2	AGCTCAGACAGCTCTTACCAC	CAAATTCTCTTTCTGGTCCCC
INHBB	NM_002193.3	ATCAGCTTCGCCGAGACA	GCCTTCGTTGGAGATGAAGA
GAPDH	NM_002046.3	CGCCCCACTTGATTTTGG	ATGGATCCCATCACCATCTT

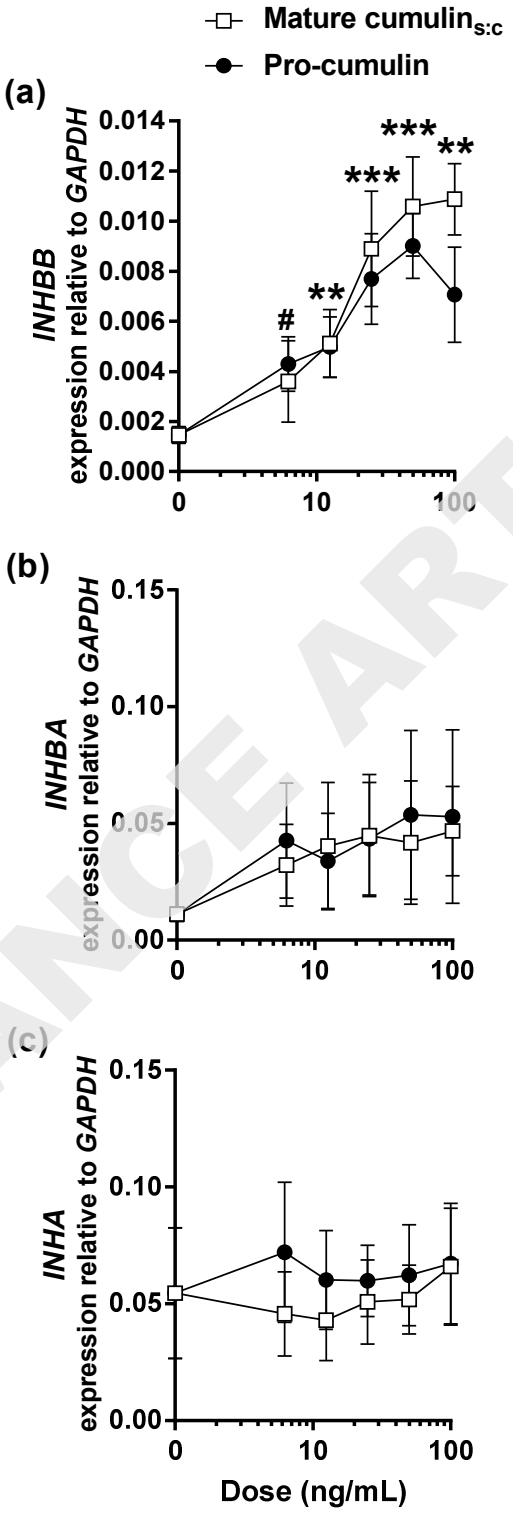
ADVANCE ARTICLE: Endocrinology
ENDOCRINE SOCIETY
ADVANCE ARTICLE
Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

ADVANCE ARTICLE: Endocrinology

ADVANCE ARTICLE

Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

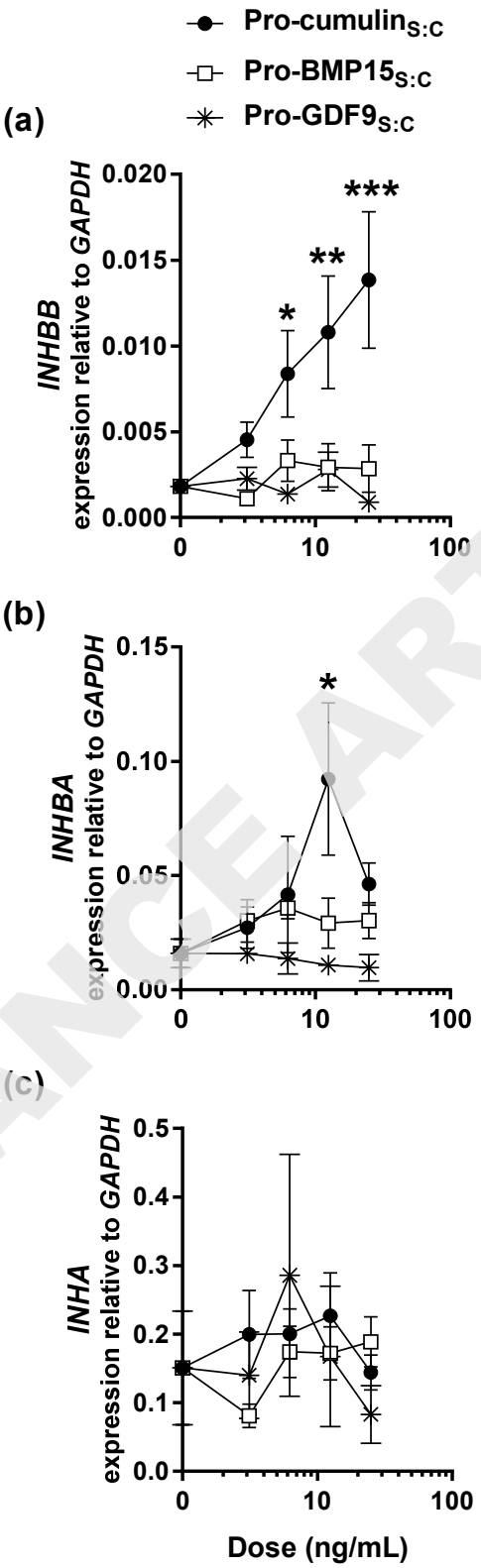
ADVANCE ARTICLE: Endocrinology



ADVANCE ARTICLE

Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

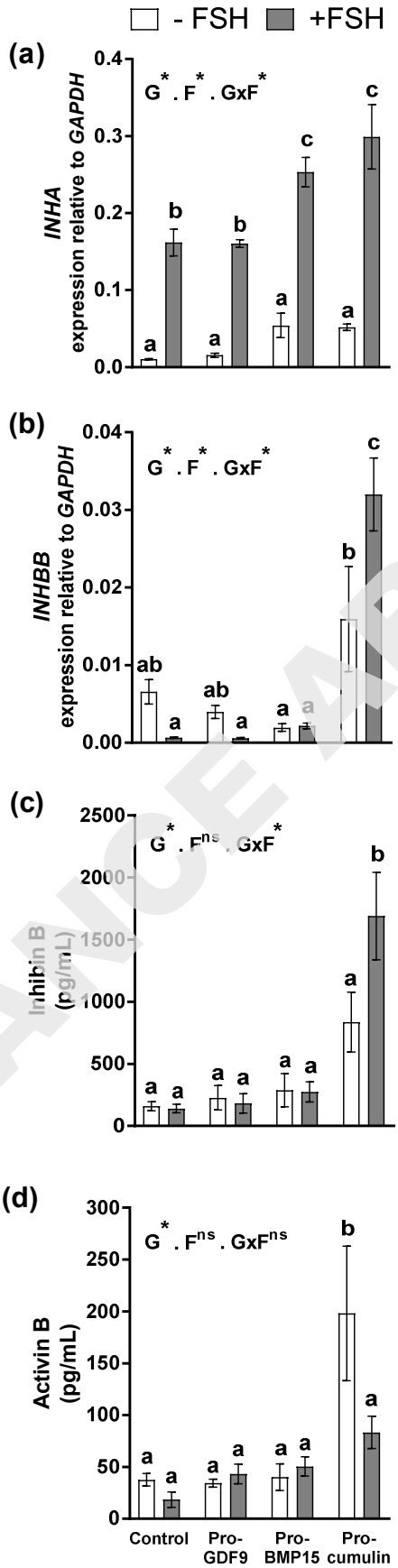
ADVANCE ARTICLE: Endocrinology



ADVANCE ARTICLE

Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

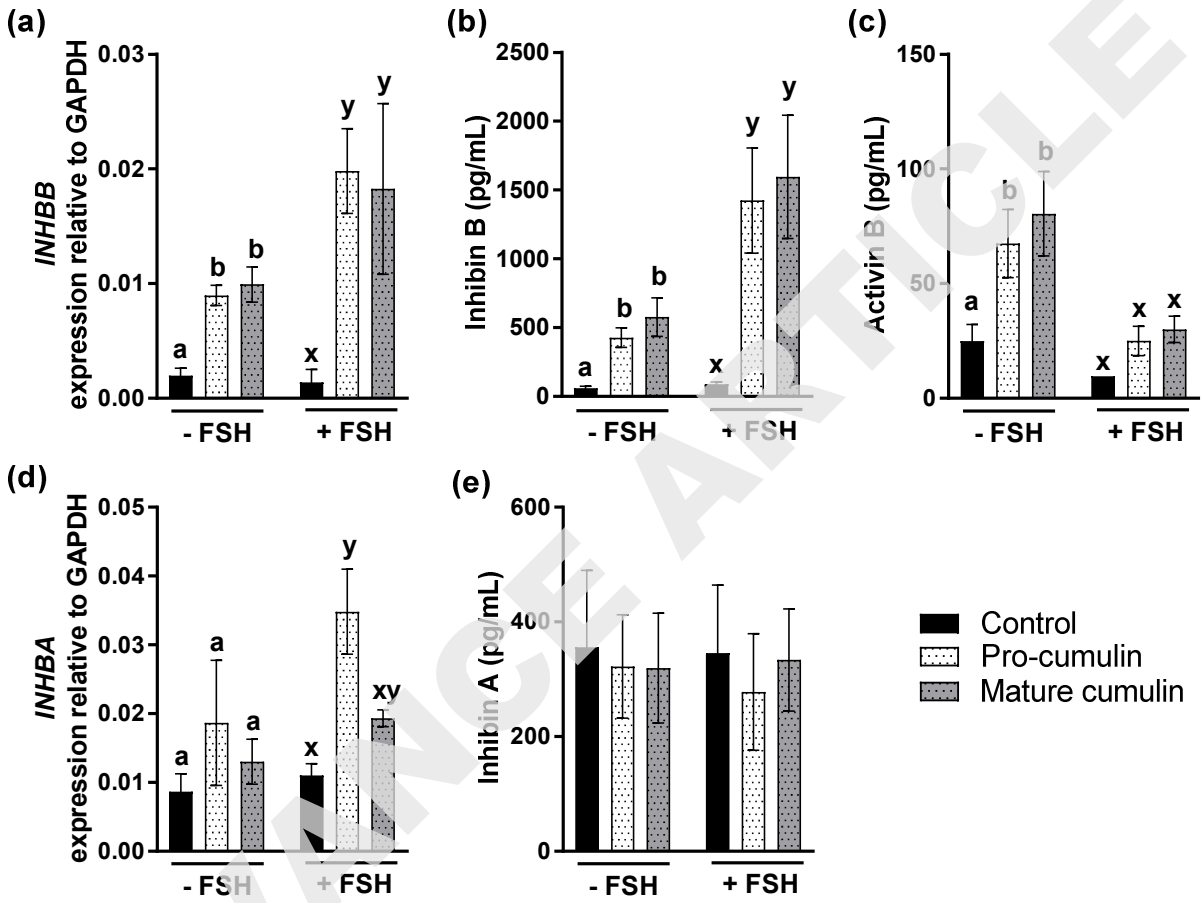
ADVANCE ARTICLE: Endocrinology



ADVANCE ARTICLE

Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019

ADVANCE ARTICLE: Endocrinology



ADVANCE ARTICLE

Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-01026/5308413 by University of Keele user on 13 February 2019